CHAPTER TWO

Genetic Variants of the Milk Proteins

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I. INTRODUCTION

A. General Characteristics of Milk Proteins

Studies on the milk protein system have been in progress for nearly 100 years. However, the last decade has brought some of the most interesting discoveries regarding these protein families. We feel that a brief review of the milk proteins is worthwhile. About 80% of the total milk protein occurs as casein. Caseins are that unique group of phosphoproteins, neatly packaged for secretion from the lactating cell, in a form termed the casein micelle. Waugh and von Hippel (1956) were the first to demonstrate that casein micelles (composed of α_{s1}-, β- and κ-caseins) were held in colloidal suspension by a protective colloid termed κ-casein.

The stability of this colloid can be weakened or destroyed by a number of factors: addition of acid to pH 4.6, addition of excess Ca^{2+} or by the action of rennin. The major components of the casein micelle, the α_{s1}- and β-caseins, are hydrophobic for the most part, but can be distinguished by their amino acid composition, phosphorus content, association behavior, sensitivity to Ca^{2+}, and distinct mobilities upon gel electrophoresis. κ-Casein, the lesser of the three main components in concentration, is more hydrophilic and insensitive to Ca^{2+}, is low in phosphorus (0.15%), contains carbohydrate, and is attacked by rennin during the primary phase of reaction with this enzyme.

The whey proteins, β-lactoglobulin (β-Lg), α-lactalbumin (α-La), the immune globulins, and bovine serum albumin (BSA) are soluble at pH 4.6 and also remain in solution when the caseins are centrifuged from suspension. These proteins have been the subject of much research. Suffice it to say that they, unlike the caseins, have ordered structures; β-Lg and α-La can be easily crystallized. The whey proteins are heat denaturable; accordingly their role in the gelation of concentrated milks has been a subject of considerable investigation.

For more extensive reviews of the chemistry of the milk proteins see Farrell and Thompson (1973) and Thompson (1971).

B. Methods of Detection of Milk Protein Polymorphs

Detection of the genetic polymorphs of the whey proteins and caseins is a relatively simple task. The earliest methods of their detection were based on zonal paper electrophoresis (Aschaffenburg and Drewry, 1955; Aschaffenburg, 1961). While these methods lacked the resolving power of more recently adopted ones, they clearly revealed that proteins from the milks of individual cows differed and that these differences had a genetic basis. Polyacrylamide gel electrophoresis at pH 9.1 (Table I), while excellent for the detection of α_{s1}-, κ-, and β-casein variants, left much to be desired for detection of subtle differences in β-casein A (β-Cn^A). It was necessary to supplement alkaline gel electrophoresis at pH 9.1 with gel conditions at pH 2.8 where striking differences in the heterogeneity of β-Cn^A was revealed. A detailed review of methods of phenotyping the milk proteins (Thompson, 1970) illustrates a variety of methods now in use. However, Fig. 1 serves to show the utility of starch-gel electrophoresis in detecting variations in the whey proteins and caseins, simultaneously.

TABLE I

RELATIVE MOBILITIES OF α_{s1}- AND β-CASEIN VARIANTS BY STARCH-GEL AND POLYACRYLAMIDE-GEL ELECTROPHORESIS[a]

Variant	Starch	Acrylamide
α_{s1}-A	1.18	1.22
α_{s1}-D	1.13[b]	1.15[c]
α_{s1}-B	1.10	1.13
α_{s1}-C	1.07	1.10
α_{s2}-(zone 1.04)	1.04	1.03
α_{s3}-(reference zone)	1.00	1.00
β-A[d]	0.80	0.65
β-B	0.76	0.61
β-D	—	0.58
β-C	0.70	0.54

[a] Using the zone reference system of Wake and Baldwin (1961). All mobilities except those designated otherwise are from Thompson *et al.* (1964).

[b] de Koning and van Rooijen (1967).

[c] Grosclaude *et al.* (1966).

[d] Alkaline gel electrophoresis only.

II. MOLECULAR BASIS FOR MILK PROTEIN VARIATIONS

A. Substitutions, Deletions, and Additions in DNA

The basis for mutations in mammalian and nonmammalian species is becoming more clearly understood with each passing year. The type of protein biosynthesized will be determined largely by the DNA contained in the nucleus of each cell, a part of which is responsible for the production of messenger ribonucleic acid (mRNA). Simply stated, mRNA travels from the nucleus of the cell to the site of protein synthesis, the ribosomes, where in combination with transfer RNA (tRNA) and ribosomal RNA (rRNA) the coded message of mRNA is transcribed. Under usual circumstances one would expect that the mRNA "type" would be transcribed at least three times.

Mutations in proteins arise when changes in the sequence of the base pairs of the DNA occurs. The frequency of this change is 10^{-4} to 10^{-5}. Hence the probability of a mutation is quite low if one excludes the possibility of "hot spots" (mutable regions) within the DNA strand. Changes in base pairs of DNA can occur in three different ways: (1) The first is a simple change in one of the bases which results in the sub-

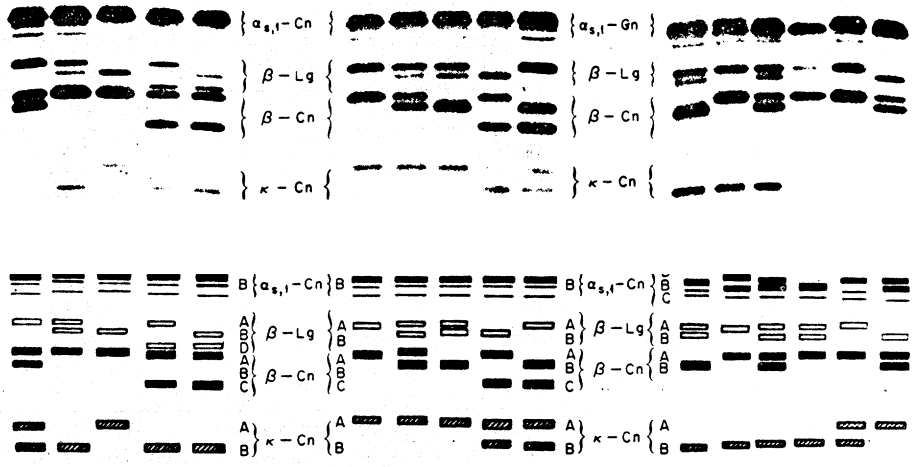


Fig. 1. Simultaneous phenotyping of caseins and β-lactoglobulins by thin starch-gel electrophoresis at pH 8.4, borate buffer in the presence of mercaptoethanol. Gel patterns courtesy of Dr. Wieslaw Michalak, Warsaw, Poland.

stitution of one amino acid for another in the protein molecule and is predicted by the triplet code (for example, glycine for glutamic acid). Many base pair substitutions are reversible; therefore, a "back" mutation to the wild type is common. However, this does not seem to be the situation with the bovine milk proteins. Mutations of the substitution type (Table II), while reflecting changes in the duplication process, may also arise from chemical and/or photochemical mutagens. (2) The second type of mutation (deletion of base pairs) arises less frequently than simple base substitution. It has been hypothesized (Watson, 1970) that during meiosis, chromosomes under physical stress may break. Upon recombination, the chromatids may not create two reciprocally recombinant chromatids but, rather, cross-over products which have a segment of DNA deleted. Of course, the segment deleted may range from a single base pair to as many as 39 in the case of α_{s1}-casein A (Thompson *et al.*, 1969). Consequently, mRNA produced from this gene will be responsible for the biosynthesis of an α_{s1} molecule with distinctly different characteristics from the B or C molecules (see below). (3) The third type of mutational event is the insertion of one or more base pairs in the gene. This process occurs in a manner similar to deletion except that upon breakage and union of chromatids, a segment of DNA is added to the cross-over products. To date, this type of mutation has not been observed in any of the milk proteins.

Conjugated proteins, such as the phosphorylated caseins, are of par-

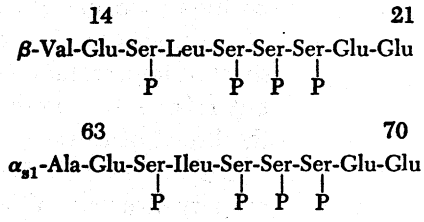
TABLE II

ASSIGNMENT OF mRNA TRIPLET CODONS TO SOME OF THE MORE COMMON MILK PROTEIN VARIANTS

Protein	Amino acid substitution	Possible codons[a]
α-Lactalbumin		
B → A	Arg → Gln	$CG^{A/G} \rightarrow CA^{A/G}$
β-Lactoglobulin		
B → C	Gln → His	$CA^{A/G} \rightarrow CA^{U/C}$
B → D	Glu → Gln	$GA^{A/G} \rightarrow CA^{A/G}$
B → A		
1.	1. Gly → Asp	$GG^{U/C} \rightarrow GA^{U/C}$
2.	2. Ala → Val	$GC^{U/C/A/G} \rightarrow CU^{U/C/A/G}$
α_{s1}-Casein		
B → C	Glu → Gly	$GA^{A/G} \rightarrow GG^{A/G}$
B → D	Pro → Ser	$CC^{U/C/A/G} \rightarrow UC^{U/C/A/G}$
κ-Casein		
B → A		
1.	1. Ala → Asp	$GC^{U/C} \rightarrow GA^{U/C}$
2.	2. Ileu → Thr	$AU^{U/C/A} \rightarrow AC^{U/C/A}$

[a] Possible mRNA codons obtained by Nirenberg *et al.* (1965) from *Escherichia coli* studies.

ticular importance from the point of view of mutations. It will be recalled (Bingham *et al.*, 1972) that the newly biosynthesized casein molecule is phosphate and carbohydrate free, these two being added after protein synthesis. However, the substitution of one amino acid for another, in a critical region of the molecule, may alter the specificity of the phosphorylating enzyme, caseinkinase. According to Mercier *et al.* (1971), a particular sequence of amino acids must exist for the caseins to be phosphorylated. A serine or threonine residue may be phosphorylated if a glutamic acid or another phosphorylated residue is two residues to its right in the sequence as follows:



The top partial sequence is that of β-casein while the lower is that of α_{s1}-casein. Note the relative positions (21 and 70) of glutamic acid in

relation to the phosphorylated serine residues. Of interest is the α_{s1}-casein variant D. In this protein a threonine is substituted for an alanine residue. Theoretically, the D form of the protein should be indistinguishable from the B form by gel electrophoresis—it is not. The reason for this is clear according to the theory of Mercier *et al.* (1971): a glutamic acid residue (position 55) is two removed from the threonine residue which has replaced the alanine; thus, it is phosphorylated and the additionally charged D variant is distinguishable from the B variant by electrophoresis at alkaline pH's.

B. Placement of Mutations within the Protein Molecules

Until recently, the primary structure of the milk proteins was unknown. The first to be totally sequenced was the relatively small molecule, α-lactalbumin, which contains 123 amino acid residues (Brew *et al.*, 1970). Two variants of the protein are known, A and B, the former of which is restricted to the milks of *Bos indicus* cattle. The only difference between the two variants is the substitution of glutamic acid in position 10 for arginine. Electrophoretic mobility differences are clearly seen on the basis of this charge difference. There is no suggestion that the substitution of glutamic acid for arginine has affected the role of α-lactalbumin in the biosynthesis of lactose. One could speculate, however, that a critical mutation in such a functional protein would impair lactose synthesis to the extent that normal milk secretion would not occur.

Of the principal whey proteins, β-lactoglobulin is the least characterized with regard to primary structure. McKenzie (1971) has published a partial primary structure (Fig. 2) of the β-LgA molecule which duplicates the effort of Frank and Braunitzer (1967). They report that β-LgB has an alanine residue at position 69 and a glycine at either positions 121 or 122. β-Lg C is similar to B but has histidine at either positions 115 or 116. A Droughtmaster β-Lg has the same amino acid composition as β-A but contains carbohydrate. This variant, in the strictest interpretation of "mutation," does not arise from a coding change, but reflects the presence of a carbohydrate adding enzyme peculiar to the breed of cattle.

Caseins, of the α_{s1} series, are remarkably well characterized as to primary structure, illustrated in Fig. 3 (Mercier *et al.*, 1971; Grosclaude *et al.*, 1972). Suffice it to say that the research of the French workers was a monumental contribution to molecular biology, in general, and milk protein chemistry, in particular. The amino acid substitutions in the

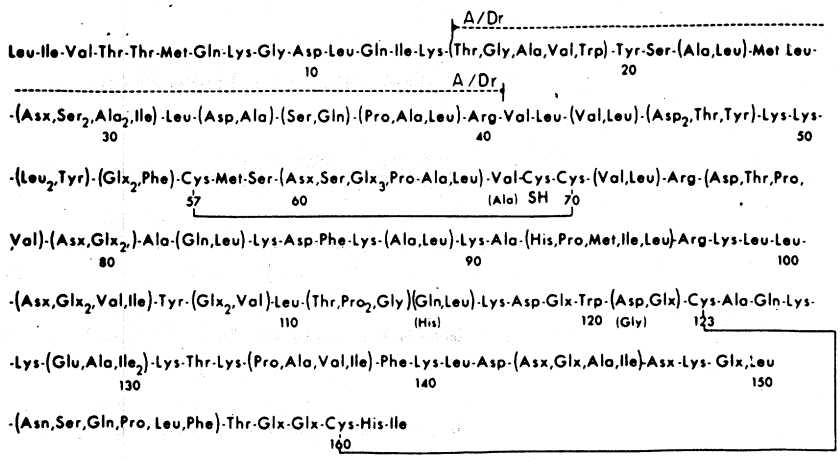


Fig. 2. Partial amino acid sequence of β-lactoglobulin A (Frank and Braunitzer, 1967) as modified by McKenzie (1971).

β-caseins (Grosclaude *et al.*, 1972; Ribadeau-Dumas *et al.*, 1972) are compared to β-A^2 (Fig. 4); β-A^1, β-B, and β-C all differ by a 67 Pro-His substitution. In β-B an additional substitution is the replacement of serine by arginine in position 122. β-C contains lysine at position 37 instead of glutamic acid. Using the theory of Mercier *et al.* (1971) (mentioned above), a substitution for Glu in position 37 would hinder phosphorylation of serine at position 35. This is, in fact, the case as evidenced by the lower phosphorus content in β-C. Table II summarizes the assignment of mRNA triplet codons to some of the milk protein variants mentioned previously.

III. SIGNIFICANCE OF POLYMORPHISM

A. Occurrence and Breed Specificity

The serendipitous discovery of genetic polymorphism in α_{s1}-casein by Thompson *et al.* (1962) has led to a fruitful study of the genetics and chemistry of the polymorphs. To date, four variants of the α_{s1} series (locus symbol α_{s1}-Cn) are known to exist. They are termed A, B, D, and C (in order of decreasing electrophoretic mobility) and their synthesis is controlled by four allelic autosomal genes with no dominance. The phenotypes correspond to the genotypes; i.e., A(A/A), AB(A/B), AC(A/C), etc.

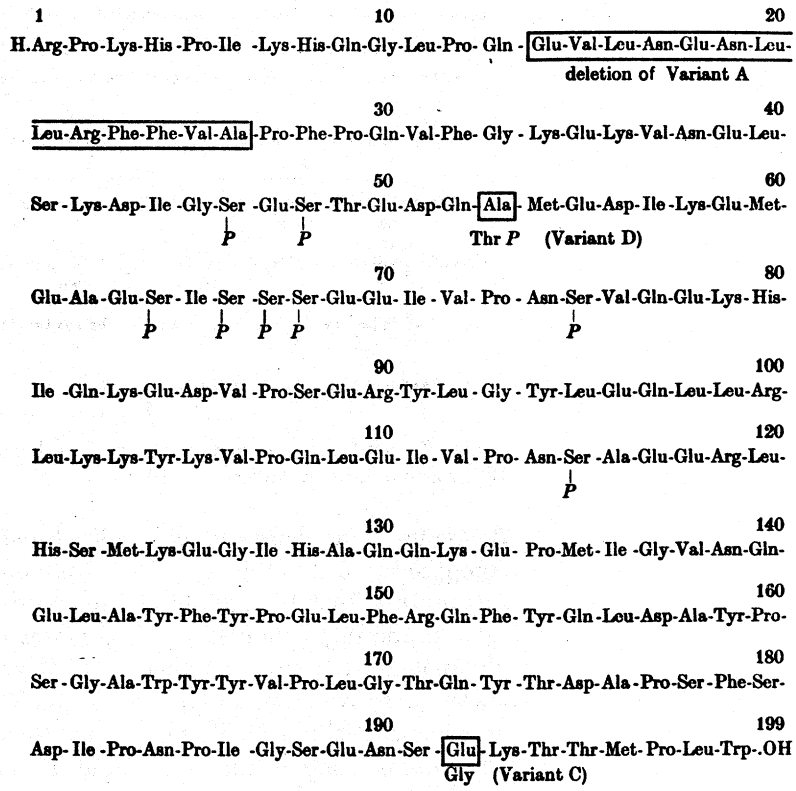


Fig. 3. The primary structure of α_{s1}-casein B. (Mercier *et al.*, 1971.)

Several interesting aspects have emerged from the study of casein polymorphism. The synthesis of specific α_{s1}-casein polymorphs is breed specific (Table III). This aspect will be discussed in considerable detail. Polymorphism of α_{s1}-casein is not universal in Western dairy cattle breeds (*Bos taurus*); Ayrshire and Shorthorn cattle (Aschaffenburg *et al.*, 1968), for example, possess genes for control of synthesis of α_{s1}-Cn^B only. Guernsey and Jersey cattle, on the one hand, possess genes for the control of both α_{s1}-Cn^B and α_{s1}-Cn^C; α_{s1}-Cn^B predominates in both breeds, but Jersey show a higher gene frequency of the C variant. Holstein cattle, on the other hand, also synthesize B and C (as well as A) but show a low gene frequency (0.05) for the C variant.

Thymann and Larsen (1965) reported an extensive survey (over 2000 cattle) of milk protein polymorphism in Danish cattle—RDM, SDM, and Jersey. (Incidentally, the Danish workers were the first to demon-

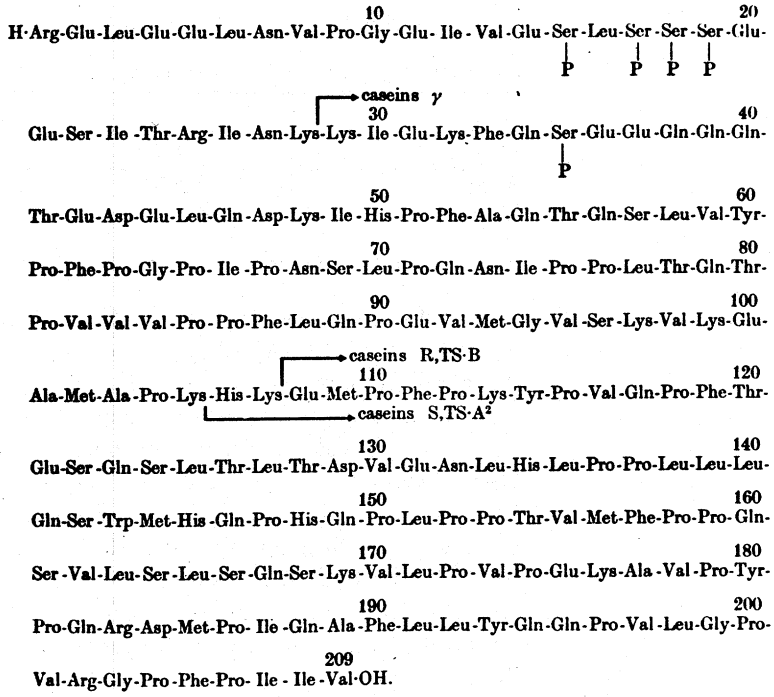


Fig. 4. The primary structure of β-casein A^2. (Ribadeau-Dumas *et al.*, 1972.)

strate by family studies that κ-casein variation is genetically controlled, although others have made unsupported suggestions that it is.) RDM and SDM synthesize essentially α_{s1}-Cn^B; Thymann and Larsen's data

TABLE III

GENE FREQUENCIES AND BREED SPECIFICITY OF α_{s1}-CASEIN VARIANTS

Breed	α_{s1}-Casein variant		
	A	B	C
Holstein	0.08[a]	0.87	0.05
Guernsey	—	0.77	0.23
Jersey	—	0.72	0.28
Brown Swiss	—	0.94	0.06
Ayrshire	—	1.00	0
Shorthorn	—	1.00	0
Zebu (Indian)	—	0.16	0.84
Boran (African)	—	0.33	0.67

[a] Gene frequency of α_{s1}-A is not representative of the breed since it seems to be restricted to one given blood line (Kiddy *et al.*, 1964).

TABLE IV

GENE FREQUENCIES IN α_{s1}-CASEIN FROM DANISH CATTLE

Breed	α_{s1}-Casein variant		
	A	B	C
RDM	0.005	0.98	0.01
SDM	0	1.00	0.004
Jersey	0	0.95	0.05

(Table IV) on Jersey cattle, α_{s1}-Cn^C (0.05), differ from those reported by Kiddy *et al.* (1964) for Jersey cattle bred in the United States. While the gene frequencies for β-casein agree between both groups, it is not unexpected that the gene frequency of a particular polymorph (in this case, α_{s1}-Cn) will vary from one herd or location to another as has often been observed. Cuperlovic *et al.* (1964) observed in 59 Yugoslavian and Hungarian cattle that α_{s1}-Cn^B predominates (0.88) when they examined the caseins of Simmentaler, Frisisk × Simmentaler, RDM × Busa, and Busa. Sandberg (1967) examined the milk of 193 Swedish red and white (SRB), 320 Swedish Friesian (SLB) and 85 Swedish polled (SKB) and found little variation in gene frequencies of α_{s1}- and β-caseins among the three breeds.

Aschaffenburg *et al.* (1968) examined the caseins of Indian and African Zebu cattle (*Bos indicus*) for casein polymorphism. Interestingly α_{s1}-Cn^C predominates in these cattle; contrast this with Western cattle, and a significant difference is evident. The gene frequencies of six breeds in Indian Zebu cattle are shown in Table V, the difference in gene frequency of α_{s1}-Cn^C in *Bos indicus* as compared with *Bos taurus* being clearly demonstrated (Table III). The good agreement of observed phenotypes of Zebu cattle with expected values from

TABLE V

GENE FREQUENCIES OF α_{s1}-, β-, AND κ-CASEINS IN BREEDS OF ZEBU CATTLE[a]

Breed[b]	α_{s1}		β			κ	
	B	C	A	B	D	A	B
Hariana (72)	0.21	0.79	0.79	0.21	0	0.79	0.21
Sahiwal (21)	0.05	0.95	0.98	0.02	0	0.90	0.10
Tharparker (7)	0.14	0.86	0.86	0.14	0	0.86	0.14
Deshi (56)	0.06	0.94	0.96	0.05	0.03	0.82	0.14
Girand Red Sindhi (5)	0	1.00	1.00	0	0	1.00	0

[a] Using 161 samples.

[b] Number of samples for each indicated in parentheses.

TABLE VI

A REPRESENTATIVE PATTERN OF INHERITANCE OF α_{s1}-, β-, AND κ-CASEINS[a]

Caseins	Observed	Expected
α_{s1}-Caseins		
B	3	2.4
BC	28	29.3
C	91	90.3
β-Caseins[b]		
A	92	90.3
AB	25	29.3
B	3	2.4
κ-Caseins[b]		
A	95	95.7
AB	33	31.7
B	2	2.6

[a] Hardy-Weinburg expectations; all Zebu breeds.

[b] Includes eight samples in addition to those tabulated for α_{s1}-caseins.

Hardy-Weinberg calculations are shown in Table VI. κ-Casein A predominates in Indian Zebu (as well as African Zebu) and doubtless it is inherited in a straightforward Mendelian manner as shown by daughter-dam comparisons and Hardy-Weinberg expectation (Table VI). To date, Jersey cattle are the only breed in which κ-casein B predominates.

Grosclaude *et al.* (1966) reported the occurrence of a new α_{s1}-casein variant, α_{s1}-Cn^{D}, in French Flemande cattle. In this breed they found gene frequencies for B, C, and D of 0.87, 0.09, and 0.04, respectively. The D variant has also been observed by Dr. W. Michalak in Polish cattle and may yet be observed in other breeds of cattle.

Kiddy *et al.* (1964) reported that α_{s1}-Cn^{A} is restricted to the Holstein breed and, in fact, considered that the mutation may be a relatively new one. Certainly, in the United States at least, this variant has become reasonably widespread due to (a) artificial breeding and (b) its association with high-producing cattle. The Danish workers Thymann and Larsen (1965) suggested, however, that the α_{s1}-Cn^{A} variant is not restricted to American Holsteins and does, in fact, occur in RDM cattle (gene frequency, 0.005). However, since the purported A was not compared with authentic A, further proof of identity was needed. Recent studies by Farrell *et al.* (1971) on chymotryptic "fingerprints" of authentic A compared with Danish A show the two to be identical. The frequency of the A variant in American Holsteins, which have been examined, is 0.08. The author has examined a single casein sample from a

New Zealand Friesian cow, supplied by Dr. L. Creamer, which was phenotyped α_{s1}-AB. Doubtless, the A variant will be observed elsewhere and new variants of the α_{s1} series will be reported.

The brilliant studies of Aschaffenburg (1961) were the first reported on genetic variation in any of the caseins. He clearly demonstrated breed specificity of the occurrence of β-caseins A, B, and C (locus symbol, β-Cn), of which A is common to all breeds of dairy cattle. β-Casein C (Table VII) has been observed in Guernsey (Aschaffenburg, 1961), Brown Swiss (Thompson *et al.*, 1964) and Hungarian and Yugoslavian dairy cattle (Cuperlovic *et al.*, 1964). In Yugoslavian cattle (Simmentaler, Frisisk × Simmentaler, and Busa) the gene frequency is low (0.005), whereas in Hungarian cattle it is 0.10 or close to that of Guernsey (0.16). β-Casein B is fairly common in Jersey, Brown Swiss, and Indian Zebu as is shown in Table VII, but its frequency is low in Hungarian and Yugoslavian cattle.

β-Casein D, observed in Indian and African Zebu (Aschaffenburg *et al.*, 1968), has not been observed in Western breeds of cattle. It has, however, been observed only in low frequency (0.02) in a few of the many breeds of Zebu cattle.

Although detailed reports on the polymorphs of γ-, TS-, R and S caseins could be reported, we limit our comments to the observation (Gordon *et al.*, 1972) that the variants of these minor components arise as a result of proteolytic cleavage of the parent molecule, β-casein.

B. Linkage of Genes

King *et al.* (1965) and Grosclaude *et al.* (1964) concurrently but independently reported a close correlation between the loci controlling

TABLE VII

GENE FREQUENCIES AND BREED SPECIFICITY OF β-CASEIN VARIANTS

Breed	By alkaline gel electrophoresis				By acid gel electrophoresis[a]
	β-A	β-B	β-C	β-D	
Holstein	0.98	0.02	0	0	A^1, A^{2*}, A^3
Guernsey	0.98	0.004	0.16	0	A^1, A^{2*}
Jersey	0.62	0.38	0	0	A^1, A^{2*}
Brown Swiss	0.79	0.19	0.02	0	A^1, A^{2*}
Ayrshire	1.00	0	0	0	A^{1*}, A^2
Shorthorn	1.00	0	0	0	—
Zebu (India)	0.85	0.13	0	0.02	A^1, A^{2*}
Boran (Africa)	0.93	0.05	0	0.02	—

[a] Asterisk denotes form of β-casein predominating by acid gel electrophoresis.

α_{s1}-Cn and β-Cn polymorphism. Certain combinations of α_{s1}-Cn and β-Cn are common (i.e., α_{s1}-Cn^{B}, β-Cn^{A}; α_{s1}-Cn^{BC}, β-Cn^{A}), while others (α_{s1}-Cn^{BC}, β-Cn^{B}; α_{s1}-Cn^{C}, β-Cn^{AB}; and α_{s1}-Cn^{C}, β-Cn^{B}) occur infrequently if at all, and are considered forbidden combinations. The notable absence of these last three classes is observed with Jersey cattle as shown in the contingency table (Table VIII).

Patterns similar to the Jersey breed emerge on comparing Guernsey and Brown Swiss cows; however, the expectation of observing certain combinations is admittedly small (Tables IX and X). Close linkage of the α_{s1}-Cn and β-Cn loci has also been observed in Indian and African Zebu cattle; no crossing over of loci has been observed (Aschaffenburg *et al.*, 1968). King *et al.* (1965) remarked:

> The present finding that the two kinds of variants (α_{s1}- and β-) do not occur independently suggests that the two loci might be linked, and in the Jersey herd, for example, the following chromosomes would be postulated: α_{s1}-Cn^{B}, β-Cn^{A}; α_{s1}-Cn^{B}, β-Cn^{B}; and α_{s1}-Cn^{C}, β-Cn^{A}. Since the α_{s1}-Cn^{B}, β-Cn^{A} combination prevails in all breeds, the other combinations are assumed to be the result of mutations in either α_{s1}-Cn or β-Cn. The combination of α_{s1}-Cn^{C}, β-Cn^{B} apparently does not occur, so the linkage would have to be very close unless recombinant types were at a selective advantage.

Grosclaude *et al.* (1964) further elaborated on the linkage of α_{s1}-Cn and β-Cn. They concluded that the loci are either identical (pleotropic) or closely correlated. If the latter hypothesis is true then the upper limit of the distance between the two loci can be estimated by the following reasoning: in effect, if x is the percentage of recombinations expressing the distance between the two loci, and n is the number of useful pairs, x being small, the number of recombinants follows a law of Poisson of parameter nx; the probability $P_{(0)}$, that recombinants will not be observed is then $P_{(0)} = e^{-nx}$. By fixing the threshold of probability at 0.05, Grosclaude *et al.* (1965) found x to be 0.038. The distance between the two loci is then <3.8 units of recombination.

More recently, Grosclaude *et al.* (1965) have proposed a close correlation of the κ-Cn locus with the α_{s1}- and β-Cn loci. They conclude that there is a 0.95 probability that the distance between the κ-Cn locus and

TABLE VIII

PATTERN OF OCCURRENCE OF α_{s1}- AND β-CASEIN PHENOTYPES IN JERSEY COWS

	β-Casein		
α_{s1}-Casein	A	AB	B
B	60	73	56
BC	45	82	—
C	35	—	—

TABLE IX

PATTERN OF OCCURRENCE OF α_{s1}- AND β-CASEIN PHENOTYPES IN GUERNSEY COWS

	β-Casein			
α_{s1}-Casein	A	AB	AC	C
B	85	5	11	—
BC	52	2	5	—
C	—	—	—	—

the pair of α_{s1}-Cn, β-Cn loci is less than 2.8 units of recombination. Therefore, the linkage between the α_{s1}-Cn and β-Cn loci appears to be closer than with these two loci and κ-Cn. Thymann and Larsen (1965) concluded that the genes involved in the synthesis of β-, α_{s1}-, and κ-casein types are not transmitted independently. This could be explained by postulating that the genes responsible for the variations in the three caseins belong to the same locus, although close linkage of two to three loci could not be excluded.

Reasonable assurance of the linkage of genes controlling the synthesis of the major milk proteins (α_{s1}-, β-, and κ-caseins) adds to the growing number of examples of genetically linked synthesis of chemically related proteins; i.e., β- and δ-chain hemoglobin variants (Boyer *et al.*, 1963) and egg-white proteins.

C. Basis for Model Construction

While it remains unclear what role, if any, the genetic variants of the caseins play in casein micelle formation, we feel that current concepts proposed for micelle formation deserve mention. For the nutritional function of caseins, it is not unusual that the majority of the observed casein polymorphs have no apparent deleterious effects on the micellar system. However, α_{s1}-A represents the sequential deletion of ~4% of the α_{s1} molecule, and altered properties of milks containing this variant

TABLE X

PATTERN OF OCCURRENCE OF α_{s1}- AND β-CASEIN PHENOTYPES IN BROWN SWISS COWS

	β-Casein			
α_{s1}-Casein	A	AB	B	AC
B	146	69	4	2
BC	22	5	—	—
C	2	—	—	—

might be expected. Indeed α_{s1}-A milks are difficult to process and yield poor cheeses. The individual caseins have been studied in great detail; yet the precise structure and mechanism of formation of the casein micelle is not known. Nearly as many models have been proposed as there are investigators. Let us briefly consider why the situation exists. Electron microscopy of the casein micelles of bovine milk indicates an average diameter of ~1400 Å for the spherically shaped micelles. Thus, the volume occupied by a micelle would be in the order of $\sim 1.4 \times 10^9$ Å^3. For comparison, the β-lactoglobulin monomer occupies a volume of $\sim 2.4 \times 10^4$ Å^3. Theoretically, more than 50,000 β-lactoglobulin-like monomers could be arranged into a sphere the size of a casein micelle. Molecular weight measurements for the micelle range from 10^7 to 10^9. A speculative calculation, based on 23,000 average MW for the casein monomers $[(3\,\alpha_{s1} + 2\,\beta + 1\,\kappa)/6]$ and employing only 25,000 monomers yields a micelle molecular weight of 6×10^8. This would indicate a low density packing of the casein monomers which is consistent with the high hydrations, the random structures, and the high negative charge densities of the caseins, as compared to β-lactoglobulin. It is therefore understandable that the mechanism of assembly of this aggregate of around 25,000 monomers has not been fully elucidated. For the purpose of discussion, we shall group the various proposed models into three classes.

1. Coat-Core Models

The first class of models to be discussed actually contains two diametrically opposed theories. The model proposed by Waugh and his coworkers (Rose, 1969; Waugh *et al.*, 1970) is primarily based upon their studies of the Ca^{2+} solubilities of the caseins. The model, in essence, describes the formation of low weight ratio complexes of α_{s1}- and κ-casein in the absence of calcium. Upon addition of calcium ions, the α_{s1}- or β-caseins, which are represented by monomers with a charged phosphate containing loop, begin to aggregate to a limiting size (the caseinate core). In the presence of the low weight ratio α_{s1}–κ-complexes, precipitation of the casein is prevented by the formation of a monolayer of these low-weight α_{s1}–κ-complexes which envelops the core aggregates. This coat has the κ-casein monomers spread out on the surface and the micelle size is therefore dictated by the amount of κ-casein available. In the absence of κ-casein, the α_{s1} and β cores agglutinate and precipitate from solution. Waugh's model, as presented in Fig. 5, has a good deal of appeal since it explains the lyophilic nature of the colloidal

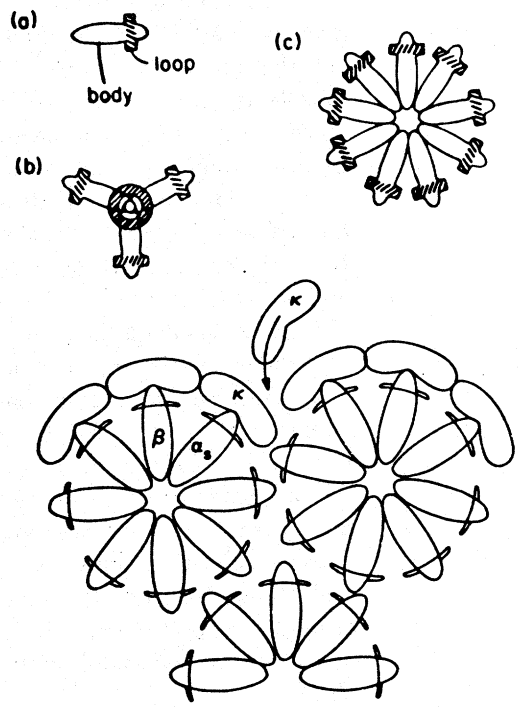


Fig. 5. Waugh's proposed model for the casein micelle: (a) monomer model of α_{s1}- or β-casein with charged loop; (b) a tetramer of α_{s1}-casein monomers; (c) planar model of a core polymer of α_{s1}- and β-caseins. The lower portion shows how κ-casein might coat core polymers. (Adapted from Rose, 1969.)

casein complex, as well as the ready accessibility of κ-casein to the enzyme rennin.

Parry and Carroll (1969) attempted to locate this surface κ-casein proposed by Waugh by use of electron microscopy. Using ferritin labeled anti-κ-casein immunoglobulins, they investigated the possibility of surface κ-casein and found little or no concentration of κ-casein on the surface of the casein micelles. Based on these results, and the size of the isolated κ-casein complex, Parry concluded that the κ-casein might serve as a point of nucleation, about which the calcium insoluble caseins might cluster and subsequently be stabilized by colloidal calcium phosphate (see Fig. 6). The action of rennin on the micelles was accounted for by demonstrating that serum κ-casein can participate in coagulation and may be involved in the formation of bridges between micelles.

The models of Parry and Waugh both predict a nonuniform distribu-

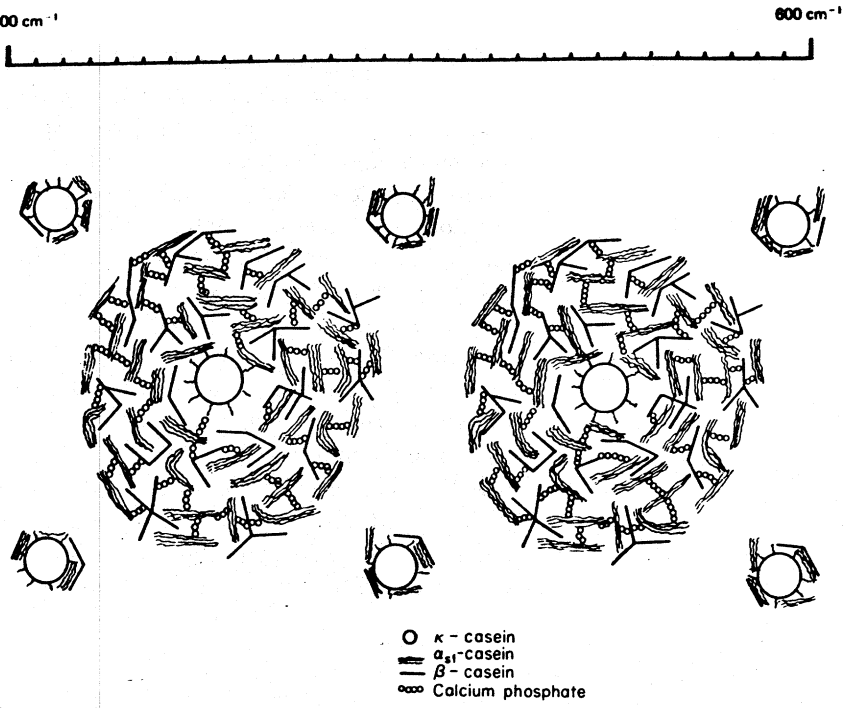


Fig. 6. Casein micelle model proposed by Parry and Carroll (1969), depicting the location of κ-casein in the micelle.

tion of κ-casein and in a sense are based upon nucleation about a core (Parry's core = κ-casein; Waugh's core = α_{s1}-, β-calcium caseinate). It is important to note that both models predict no particular stoichiometry for the casein components and demonstrate no subunit structures composed of all three casein components. Secondly, Waugh's model does not incorporate any colloidal calcium phosphate which plays an important role in casein micelle structure and stability. Finally, Ashoor *et al.* (1971) have recently demonstrated that papain, which had been cross linked by glutaraldehyde into a large insoluble polymer, caused proteolysis of all three major components of isolated casein micelles. The α_{s1}-, β- and κ-caseins were all cleaved proportionately by the enzyme super polymer. Therefore, all three components must occupy surface positions on the micelle in relatively the same proportions in which they occur in milk. This result would seem to rule out any preferential localization of κ-casein.

2. Internal Structure Models

The second class of models to be discussed are based upon the known properties of the isolated casein components, which in turn cause or direct the formation of the internal structure of the casein micelle.

Garnier and Ribadeau-Dumas (1970) have proposed a model for the casein micelle, which places a good deal of emphasis on κ-casein as the keystone of micelle structure. Trimers of κ-casein are linked to three chains of α_{s1}- and β-casein which radiate from the κ-casein node (a Y-like structure) as shown in Fig. 7. These chains of α_{s1}- and β-casein may connect with other κ nodes to form a loosely packed network. Garnier and Ribadeau-Dumas favor this type of network because it yields an open, porous structure and they have demonstrated that carboxypeptidase A with a molecular weight of ~40,000 is able to remove the C-terminal amino acids of all the casein components. The model satisfies the demonstrated porosity but places great steric restraints upon κ-casein which possesses no α-helical or other prominent secondary structures. In addition, studies by Cheeseman (1968) and others indicate that while disulfide linked trimers of κ-casein do occur, the major-

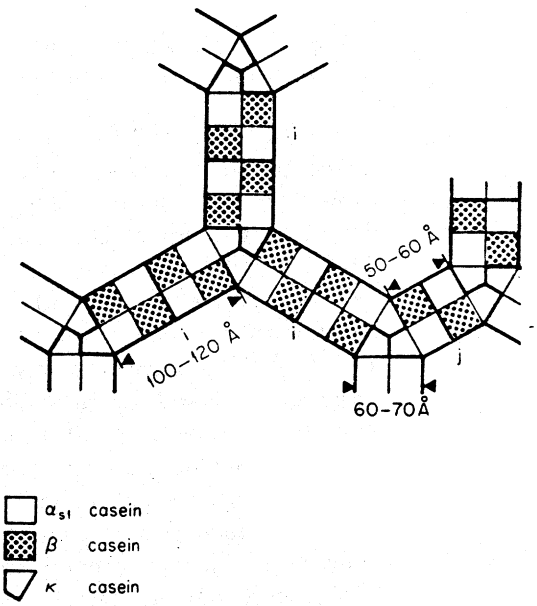


Fig. 7. Structure of the repeating unit of the casein micelle adapted from Garnier and Ribadeau-Dumas (1970).

ity of the κ-casein may form aggregates of higher, as well as lower, orders. Finally, the model assigns no definite role to calcium caseinate interactions, and ignores the possibility of colloidal calcium phosphate involvement in stabilization of the micelle.

Rose used the known endothermic polymerization of β-casein as the basis for his micelle model. In this model β-casein monomers begin to self-associate into chainlike polymers to which α_{s1}-monomers become attached (Fig. 8) and κ-casein, in turn, interacts with the α_{s1}-monomers. The β-casein of the thread is directed inward, the κ outward, but as these segments coalesce, a small amount of κ-casein is inevitably placed in an internal position. As the micelle is formed, colloidal calcium phosphate is incorporated into the network as a stabilizing agent. The model is appealing in that it accounts for the occurrence of some overall stoichiometry of the various casein components, while demonstrating the role of colloidal calcium phosphate in micelle stabilization. The choice of β-casein as the basis for micelle formation is, however, questionable since Waugh *et al.* (1970) have shown that the α_{s1}- and

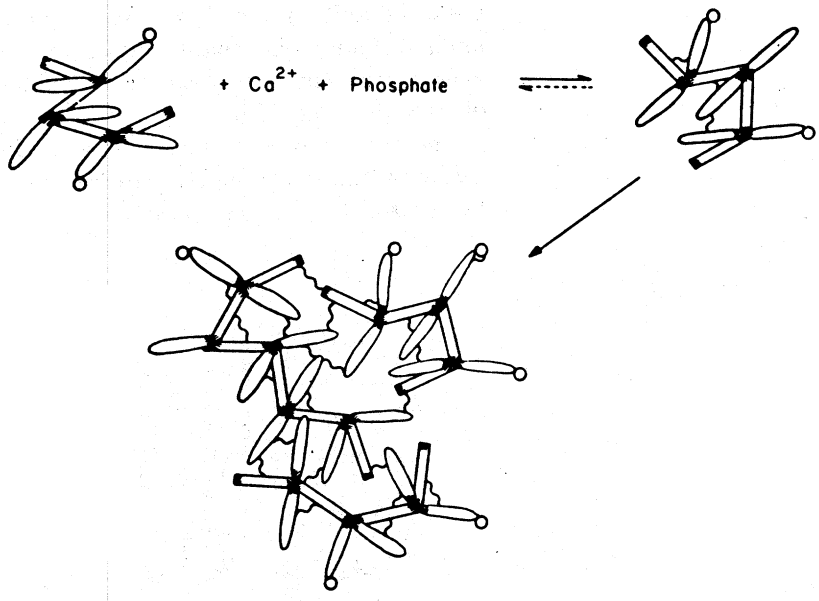


Fig. 8. Schematic representation of the formation of a small casein micelle. The rods represent β-casein, the more elliptical rods represent α_{s1}-casein, and S-shaped lines depict apatite chain formation. The circles represent κ-casein. (Adapted from Rose, 1969.)

β-caseins tend to form mixed polymers randomly; secondly, β-casein is quite structureless in solution and, finally, synthetic micelles can be formed from simple α_{s1}- and κ-casein complexes in the absence of β-casein.

3. Subunit Models

The final class of models to be discussed are those which propose subunit structure for the casein micelle. Shimmin and Hill (1964) proposed such a model based upon their study of ultrathin cross sections of embedded casein micelles by electron microscopy. They predicted a diameter of 100 Å for the subunits of the casein micelle.

Morr (1967) studied the disruption of casein micelles and proposed that the α_{s1}-, β-, and κ-monomers were aggregated by calcium into small subunits in much the same fashion as Waugh *et al.* (1970) had proposed for the entire micelle. Morr's subunits, as estimated by sedimentation velocity, have a diameter of ~300 Å. The subunits are stabilized by hydrophobic bonding and calcium caseinate bridges, and these subunits, in turn, are aggregated into micellar structure by colloidal calcium phosphate. Morr's model is summarized in Fig. 9. The average subunit size, postulated by Morr, is somewhat larger than that of Shimmin and Hill.

The hypothesis of Shimmin and Hill (1964), that sections of the casein micelles contained particles of ~100 Å diameter, was invoked by Carroll *et al.* (1970) and by Farrell and Thompson (1971) who

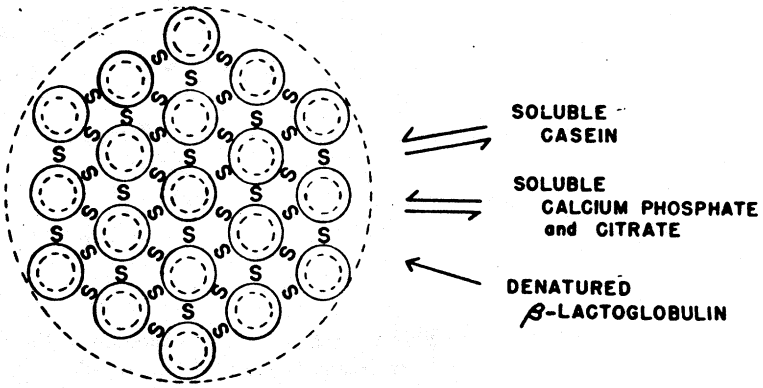


Fig. 9. Structure of the casein micelle after Morr (1967). The S-shaped lines represent calcium phosphate linkages between small spherical complexes of the α_{s1}-, β-, and κ-caseins.

observed, by electron microscopy, particles of ~100 Å diameter in the Golgi vacuoles of lactating rat mammary gland. These particles were uniform in size and may be the precursors of threadlike structures which, in turn, coalesce into the spherically shaped casein micelle (Fig. 10A, B). Subsequently, Beery *et al.* (1971) reported similar observations in bovine mammary tissue. The biosynthesis of the casein micelle from small subunits was correlated with the disruption of casein micelles by dissociating agents by Carroll *et al.* (1971). Using EDTA, urea, sodium lauryl sulfate, and sodium fluoride to disrupt micelles, the latter workers found particles of ~100 ±20 Å diameter and they noted that micelle assembly from subunits should lead to a rather uniform distribution of the α_{s1}-, β-, and κ-caseins both on the surface and in the interior of the casein micelle. Schmidt and Buchheim (1970) dialyzed milk free of calcium in the cold and also used high pressure to disrupt casein micelles; in both cases they obtained subunits of 100 Å diameter. Subsequently, Pepper (1972) reported a Stokes radius of ~50 Å for first cycle (Ca^{2+} free) casein as determined by gel filtration. The first cycle casein, after gel filtration, contained, qualitatively, all of the major casein fractions. The question yet to be resolved is whether or not the casein subunits observed by all of the above workers exhibit any stoichiometry in terms of their α_{s1}-, β-, and κ-casein content.

It has long been recognized that at least the α_{s1}- and κ-casein components occur in close association in the "α-casein complex," with β-casein being more loosely connected to the micellar complex. Furthermore, the total micellar casein exhibits an overall ratio of 3 α_{s1} : 2 β : 1 κ-casein. The apparent uniformity of first cycle (Ca^{2+} free) casein and the subunits of the Golgi vacuoles would argue in favor of some consistent stoichiometry, but there exists the reported correlation between micelle size and κ-casein content which would argue against uniform subunit composition. Thus, the existence of some type of subunit structure appears certain and the question to be decided now is the nature of these subunits. From the biosynthetic point of view, the buildup of the micelle from subunits is quite attractive, as it brings the casein components into the region of assembly with minimal interactions. Addition of calcium ions could cause the polymerization of casein subunits into longer chains and these chains could be stabilized into micellar spheres by the deposition of colloidal calcium phosphate. The assembly of the casein micelle from preformed subunits need not be as specific as that of tobacco mosaic virus, but the analogy is worthy of consideration. In the latter case, the structured

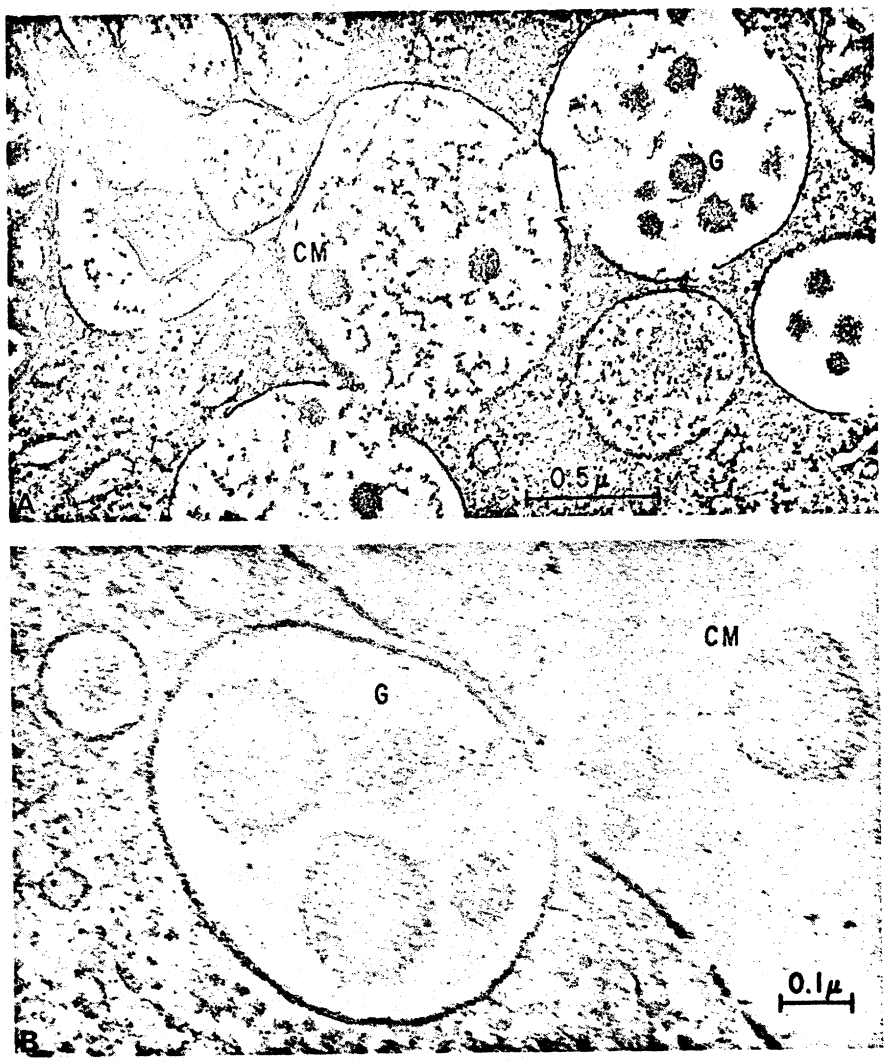


Fig. 10. A, Formation of casein micelles (CM) within Golgi vacuoles (G) of lactating rat mammary gland. Initially, threadlike structures with some degree of periodicity appear, then more compact micelles seem to occur. Sections of the gland were fixed in buffered OsO_4, Epon embedded, and stained with uranyl acetate and lead citrate. (Carroll *et al.*, 1971.) B, A Golgi vacuole about to discharge its contents into the alveolar lumen. The Golgi vacuole shown appears to impinge upon the plasma membrane. A casein micelle is already present in the lumen. (Carroll *et al.*, 1971.) In the scale 1 μ = 1 μm.

RNA core of the virus plays a vital role in directing the correct particle assembly, whereas in the case of the micelle, amorphous apatite may serve this function. In attempting to solve the problem of casein micelle structure, it should be borne in mind that the biological function of the micelle is efficient nutrition. Hence, the interactions which yield this product, the casein micelle, need not be as specific as those which result in the formation of a virus.

IV. BIOLOGICAL FUNCTIONS OF THE MILK PROTEINS

Ebner (see Chapter 3, Vol. II, this treatise) has already shown that α-lactalbumin has a distinct biological function. We feel that β-lactoglobulin is also biologically important although we don't know exactly how or why. In our laboratory we have observed (Farrell and Thompson, 1971) that β-lactoglobulin produces a 35–40% inhibition of the hydrolysis of *p*-nitrophenyl phosphate (*p*-NPP) and *o*-carboxyphenyl phosphate by bovine spleen phosphoprotein phosphatase. Base denaturation of the β-lactoglobulin molecule markedly decreases the degree of inhibition. Other proteins (lactoferrin, γ-globulin, and serum albumin) do not inhibit the reaction. Kinetically, the inhibition of the phosphatase is defined as competitive, with a K_i equal to 1.98×10^{-5} *M*. The three genetic variants of bovine β-Lg A, B, and C were tested for their ability to inhibit the action of phosphoprotein phosphatase on *p*-NPP. The results indicate that β-Lg A > B > C in their inhibitory effect. These differences are related to the structural changes induced by the genetic substitutions. The physical aggregation of β-Lg A does not occur to any great extent under the conditions of the enzymatic assay of phosphoprotein phosphatase. However, the carboxyl-rich region responsible for the tetramerization reaction may play a role in the inhibition of *p*-NPP hydrolysis. Since the physical properties of β-Lg C are altered by the histidine/glutamine substitution, this genetic substitution may account as well for the drastically reduced ability of β-Lg C to inhibit the hydrolysis of *p*-NPP by the spleen enzyme.

β-Lactoglobulin does not inhibit the dephosphorylation of α_{s1}-casein, the major phosphoprotein of cow's milk. Thus, the inhibition appears to be limited to low molecular weight aromatic substrates and is due, in part, to substrate binding by the β-lactoglobulin. The affinity for low molecular weight aromatic phosphates, coupled with the inhibitory effect on the enzyme has led to the speculation that β-lactoglobulin may

play a regulatory role in mammary gland metabolism, though further research is needed in this area. β-Lactoglobulin homologues from goat and swine milk also inhibit the hydrolysis of *p*-nitrophenyl phosphate by phosphoprotein phosphatase, thus, providing a type of enzymatic assay for studies in the comparative biochemistry of β-lactoglobulin.

V. A PROSPECTUS ON FUTURE RESEARCH IN MILK PROTEIN CHEMISTRY

Further research in milk protein chemistry will depend largely on the application of our current knowledge to future research. Practically, we know enormous detail regarding "milk protein component chemistry." Fundamentally, however, we know little regarding the biosynthesis, phosphorylation, and assemblage of casein components. Many questions remain to be answered. Is it possible that the caseins could be synthesized as a single macromolecule and subsequently split by enzymes? What is the role of β-lactoglobulin in the lactating gland? Could it serve as a transferase? As a part of a calcium pump? As a regulator of protein biosynthesis? What is the mechanism of casein phosphorylation?

Chapters such as this are written to express the "wisdom of the wise." No doubt, however, these reflect a collection of data without much knowledge of the truth. We maintain that we have barely scratched the surface of the complex behavior and properties of the milk system. While this alone is not an ample justification for research, it must be regarded as a prime reason for attacking the "fundamentals of dairy chemistry." Once we know what a system is composed of, we can attempt to reassemble it and to relate what we find to the demands of production and technology. If we are honest, however, we confess our ignorance and maintain that we are never sure if basic research will have applied value. But we hope so.

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